WHO 5th EDITION MANUAL FOR SEMEN ANALYSIS

COUNTING SPERM CONCENTRATION and TOTAL SPERM

Pages 33-45 (Extracted)

2.7 Sperm numbers

The total number of spermatozoa per ejaculate and the sperm concentration are related to both time to pregnancy (Slama et al., 2002) and pregnancy rates (WHO, 1996; Zinaman et al., 2000) and are predictors of conception (Bonde et al., 1998;

Larsen et al., 2000). More data correlating total sperm numbers with reproductive outcome are warranted.

The number of spermatozoa in the ejaculate is calculated from the concentration of spermatozoa, which is measured during semen evalulation. For normal ejaculates, when the male tract is unobstructed and the abstinence time short, the total number of spermatozoa in the ejaculate is correlated with testicular volume (Handelsman et al., 1984; WHO, 1987; Andersen et al., 2000; Behre et al., 2000) and thus is a measure of the capability of the testes to produce spermatozoa (MacLeod & Wang, 1979) and the patency of the male tract. The concentration of spermatozoa in the semen, while related to fertilization and pregnancy rates, is influenced by the volume of the secretions from the seminal vesicles and prostate (Eliasson, 1975) and is not a specific measure of testicular function.

Comment 1: The terms "total sperm number" and "sperm concentration" are not synonymous. Sperm concentration refers to the number of spermatozoa per unit volume of semen and is a function of the number of spermatozoa emitted and the volume of fluid diluting them. Total sperm number refers to the total number of spermatozoa in the entire ejaculate and is obtained by multiplying the sperm concentration by the semen volume.

Comment 2: The generalization that total sperm number reflects testicular sperm productivity may not hold for electro-ejaculates from men with spinal cord injury, those with androgen deficiency, or for samples collected after prolonged abstinence or partial retrograde ejaculation.

Comment 3: The term "sperm density" (mass per unit volume) should not be used when sperm concentration (number per unit volume) is meant.

Determination of sperm number comprises the following steps (which are described in detail in subsequent sections).

- Examining a well-mixed, undiluted preparation of liquefied semen on a glass slide under a coverslip, to determine the appropriate dilution and appropriate chambers to use (see Section 2.8.1). This is usually the wet preparation (see Section 2.4.2) used for evaluation of motility.
- Mixing semen and preparing dilutions with fixative.
- Loading the haemocytometer chamber and allowing spermatozoa to settle in a humid chamber.
- Assessing the samples within 10–15 minutes (after which evaporation has noticeable effects on sperm position within the chamber).
- Counting at least 200 spermatozoa per replicate.
- Comparing replicate counts to see if they are acceptably close. If so, proceeding with calculations; if not, preparing new dilutions.
- Calculating the concentration in spermatozoa per ml.
- Calculating the total number of spermatozoa per ejaculate.

2.7.1 Types of counting chamber

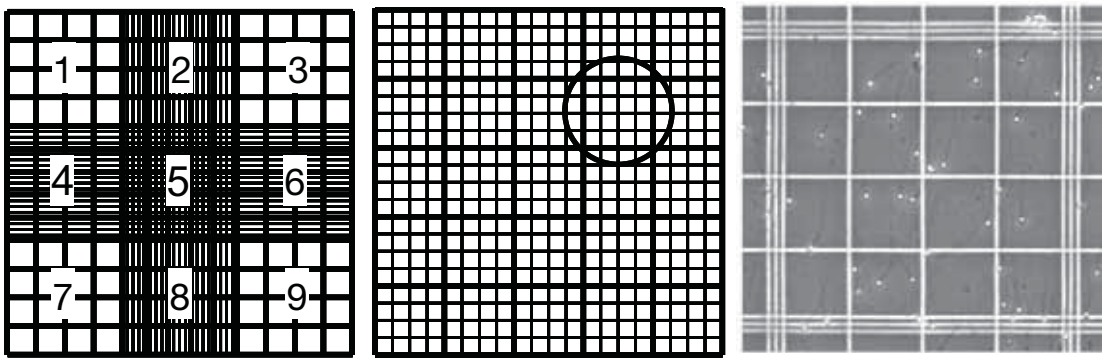
The use of 100-μm-deep haemocytometer chambers is recommended. Dilution factors for the improved Neubauer haemocytometer chamber are given here. Other deep haemocytometer chambers may be used, but they will have different volumes and grid patterns and will require different factors for calculation. Disposable chambers are available for determining sperm concentration (Seaman et al., 1996; Mahmoud et al., 1997; Brazil et al., 2004b), but they may produce different results from those of the improved Neubauer haemocytometer. Shallow chambers that fill by capillary action may not have a uniform distribution of spermatozoa because of streaming (Douglas-Hamilton et al., 2005a, 2005b). It may be possible to correct for this (Douglas-Hamilton et al., 2005a) but it is not advised (Björndahl & Barratt, 2005). The validity of these alternative counting chambers must be established by checking chamber dimensions (see Appendix 7, section A7.8), comparing results with the improved Neubauer haemocytometer method, and obtaining satisfactory performance as shown by an external quality-control programme. For accurate assessment of low sperm concentrations, large-volume counting chambers may be necessary (see Section 2.11.2).

2.7.2 The improved Neubauer haemocytometer

The improved Neubauer haemocytometer has two separate counting chambers, each of which has a microscopic 3 mm × 3 mm pattern of gridlines etched on the glass surface. It is used with a special thick coverslip (thickness number 4, 0.44 mm), which lies over the grids and is supported by glass pillars 0.1 mm above the chamber floor. Each counting area is divided into nine 1 mm × 1 mm grids. These grids are referred to by the numbers shown in Fig. 2.7.

Fig. 2.7 The improved Neubauer haemocytometer

Sketches of the inscribed area showing: all nine grids in one chamber of the haemocytometer (*left panel*); the central grid (number 5) of 25 large squares (*middle panel*); and a micrograph of part of a filled chamber *(right panel)*, showing one of the 25 squares of the central grid (the circled square in the middle panel) bounded by triple lines and containing 16 smaller squares.



Micrograph courtesy of C Brazil.

With a depth of 100 μm, each grid holds 100 nl. Four of these grids (nos 1, 3, 7 and 9) contain four rows of four squares, each holding 6.25 nl; two grids (nos 2 and 8) contain four rows of five squares, each of 5 nl; two grids (nos 4 and 6) contain five rows of four squares, each of 5 nl; and the central grid (number 5) contains five rows of five squares, each of 4 nl (Fig. 2.7, middle panel). Each of the 25 squares of the central grid (number 5) is subdivided into 16 smaller squares (Fig. 2.7, right panel). Thus, grids 1, 2, 3, 7, 8 and 9 each have four rows holding 25 nl per row, while grids 4, 5 and 6 each have five rows holding 20 nl per row.

Depending on the dilution and the number of spermatozoa counted, different areas of the chamber are used for determining sperm concentration. For 1 + 19 (1:20) and 1 + 4 (1:5) dilutions, rows from grid number 5 are assessed and, when necessary, from grids numbers 4 and 6 (see Section 2.8). For 1 + 1 (1:2) dilutions, all nine grids can be assessed if necessary to achieve a count of 200 spermatozoa (see Section 2.11.1).

2.7.3 Using the haemocytometer grid

- Count only whole spermatozoa (with heads and tails).
- Whether or not a spermatozoon is counted is determined by the location of its head; the orientation of its tail is unimportant. The boundary of a square is indicated by the middle line of the three; thus, a spermatozoon is counted if most of its head lies between the two inner lines, but not if most of its head lies between the two outer lines (Fig. 2.8, left panel).
- To avoid counting the same spermatozoon in adjacent squares, a spermatozoon with its head on the line dividing two adjacent squares should be counted only if that line is one of two perpendicular boundary lines. For example, cells may be counted if most of the sperm head lies on the lower or left centre boundaries, which form an “L” shape (see Fig. 2.8, middle panel), but not if it lies on the upper or right centre boundary line (Fig. 2.8, right panel).

Note: If there are many headless sperm tails (pinheads) or heads without tails, their presence should be recorded in the report. If considered necessary, their concentration can be assessed in the same way as for spermatozoa (see Section 2.8), or their prevalence relative to spermatozoa can be determined from stained preparations (see Section 2.17.6).

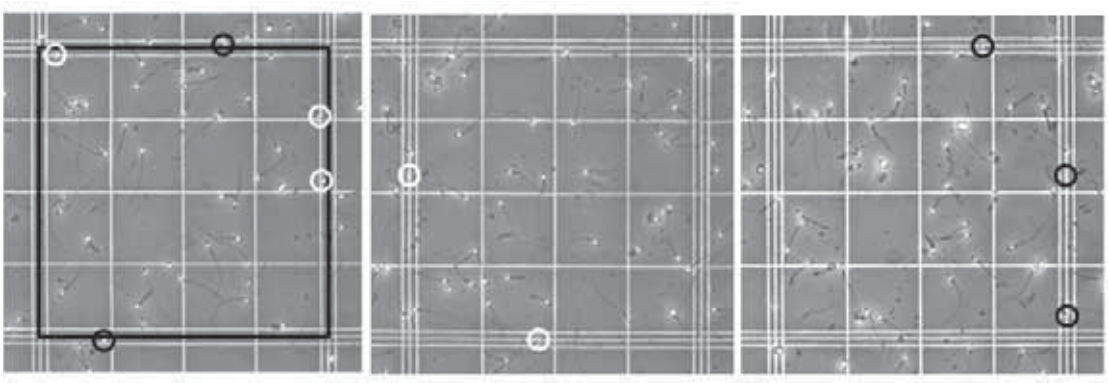
2.7.4 Care of the counting chamber

Haemocytometer counting chambers must be used with the special thick coverslips (thickness number 4, 0.44 mm).

- Clean the haemocytometer chamber and coverslip with water and dry well with tissue after use, as any dried residue can inhibit loading. Rubbing the grid surface will remove any residual spermatozoa from the previous sample.
- Soak reusable chambers and coverslips overnight in disinfectant (see Appendix 2, section A2.4) to avoid contamination with potentially infectious agents in semen.

Fig. 2.8 Which spermatozoa to count in the grid squares

The middle of the three lines defines the square's boundary (black line, *left panel*). All spermatozoa within the central square are counted, as well as those with their heads between the two inner lines (white circles), but not those whose heads lie between the outer two lines (black circles). A spermatozoon with most of its head lying on the central line is counted only if that line is the lower or left-hand line of the square (white circles, *middle panel*) but not if it is the upper or right hand line of the square (black circles, *right panel*).



Micrographs courtesy of C Brazil.

2.7.5 Fixative for diluting semen

1. Dissolve 50 g of sodium bicarbonate ($NaHCO_3$) and 10 ml of 35% (v/v) formalin in 1000 ml of purified water.
2. If desired, add 0.25 g of trypan blue (colour index 23859) or 5 ml of saturated (>4 mg/ml) gentian violet (colour index 42555) to highlight the sperm heads.
3. Store at 4 °C. If crystals form in the solution, pass it through a 0.45-μm filter before use.

2.7.6 Importance of counting sufficient spermatozoa

To reduce sampling errors, a critical number of spermatozoa have to be counted (preferably a total of at least 400, from replicate counts of approximately 200) (see Box 2.7 and Table 2.2).

Box 2.7 Errors in estimating numbers

The precision of the estimate of sperm number depends on the number of spermatozoa counted. In a Poisson distribution, the standard error (SE) of a count (N) is its square root ($\sqrt{N}$) and the 95% confidence interval (CI) for the number of spermatozoa in the volume of semen is approximately $N \pm 1.96 \times \sqrt{N}$ (or $N \pm$ approximately $2 \times \sqrt{N}$).

If 100 spermatozoa are counted, the SE is 10 ($\sqrt{100}$), and the 95% CI is 80–120 (100 ± 20). If 200 spermatozoa are counted, the SE is 14 ($\sqrt{200}$), and the 95% CI is 172–228 (200 ± 28). If 400 spermatozoa are counted, the SE is 20 ($\sqrt{400}$) and the 95% CI is 360–440 (400 ± 40).

The sampling errors can be conveniently expressed as a percentage of the count ($100 \times (\sqrt{N}/N)$). These are shown in Table 2.2.

Note: These values are only approximate, as confidence intervals are not always symmetrical around the estimate. The exact 95% confidence intervals, based on the properties of the Poisson distribution, are 361–441 for a count of 400, 81.4–121 for a count of 100, 4.80–18.4 for a count of 10, 0.03–5.57 for a count of 1, and 0.00–3.70 for a count of 0.

Table 2.2 Rounded sampling errors (%) according to total number of spermatozoa counted

Total *(N)*	Sampling error (%)	Total *(N)*	Sampling error (%)	Total *(N)*	Sampling error (%)
1	100	25	20	85	10.8
2	70.7	30	18.3	90	10.5
3	57.7	35	16.9	95	10.3
4	50	40	15.8	100	10
5	44.7	45	14.9	150	8.2
6	40.8	50	14.1	200	7.1
7	37.8	55	13.5	250	6.3
8	35.4	60	12.9	300	5.8
9	33.3	65	12.4	350	5.3
10	31.6	70	12	400	5
15	25.8	75	11.5	450	4.7
20	22.4	80	11.2	500	4.5

Comment 1: Counting too few spermatozoa will produce an uncertain result (see Appendix 7, section A7.1), which may have consequences for diagnosis and therapy (see Appendix 7, section A7.2). This may be unavoidable when spermatozoa are taken for therapeutic purposes and sperm numbers are low (see Section 5.1).

Comment 2: When semen volume is small and fewer spermatozoa are counted than recommended, the precision of the values obtained will be significantly reduced. If fewer than 200 spermatozoa are counted per replicate, report the sampling error as given in Table 2.2.

2.8 Routine counting procedure

The dilutions 1 + 4 (1:5) and 1 + 19 (1:20) are appropriate for a range of sperm concentrations, yielding about 200 spermatozoa in one or all of the haemocytometer grid numbers 4, 5 and 6 (see Table 2.3 and Box 2.8).

Box 2.8 Achieving 200 spermatozoa per replicate in the central three grids of the improved Neubauer chamber

If there are 100 spermatozoa per high-power field (HPF) of 4 nl (see Box 2.9) in the initial wet preparation, there are theoretically 25 per nl (25 000 per μl or 25 000 000 per ml). As the central grid (number 5) of the improved Neubauer chamber holds 100 nl, there would be 2500 spermatozoa within it. Diluting the sample 1 + 4 (1:5) would reduce the background and the sperm number to about 500 per grid, which is sufficient for an acceptably low sampling error.

If there are 10 spermatozoa per HPF of the wet preparation, there would be 2.5 per nl and 250 per central grid. Diluting the sample 1 + 1 (1:2) as suggested would reduce the background and the sperm number to about 125 per grid; this would give 375 in the three grids numbered 4, 5 and 6—again, this is sufficient for an acceptably low sampling error.

Note: These calculated concentrations can only be rough estimates because so few spermatozoa are counted and volumes may not be accurate. The concentrations estimated from the undiluted preparations can be between 30% and 130% of the concentrations derived from diluted samples in counting chambers.

2.8.1 Determining the required dilution

The dilution of semen required to allow sperm number to be measured accurately is assessed from an undiluted semen preparation. This is usually the wet preparation (see Section 2.4.2) used for evaluation of motility.

- Examine one of the wet preparations, made as described in Section 2.4.2, to estimate the number of spermatozoa per HPF (×200 or ×400).
- One HPF is equivalent to approximately 16 nl (at ×200) or 4 nl (at ×400) (see Box 2.9).
- If spermatozoa are observed, count them, determine the necessary dilution from Table 2.3, and proceed as in Section 2.8.2.
- If no spermatozoa are observed, examine the replicate wet preparation. If no spermatozoa are found in the second preparation, proceed as in Section 2.9.

Box 2.9 Volume observed per high-power field of a 20-μm-deep wet preparation

The volume of semen observed in each microscopic field depends on the area of the field (πr^2, where π is approximately 3.142 and r is the radius of the microscopic field) and the depth of the chamber (20.7 μm for the wet preparation). The diameter of the microscopic field can be measured with a stage micrometer or can be estimated by dividing the diameter of the aperture of the ocular lens by the magnification of the objective lens.

With a ×40 objective and a ×10 ocular of aperture 20 mm, the microscope field has a diameter of approximately 500 μm (20 mm/40). In this case, r = 250 μm, r^2 = 62 500 μm², πr^2 = 196 375 μm² and the volume is 4 064 962 μm³ or about 4 nl.

With a ×20 objective and a ×10 ocular of aperture 20 mm, the microscope field has a diameter of approximately 1000 μm (20 mm/20). In this case, r = 500 μm, r^2 = 250 000 μm², πr^2 = 785 500 μm² and the volume is 16 259 850 μm³ or about 16 nl.

Table 2.3 Semen dilutions required, how to make them, which chambers to use and potential areas to assess

Spermatozoa per ×400 field	Spermatozoa per ×200 field	Dilution required	Semen (μl)	Fixative (μl)	Chamber	Area to be assessed
>101	>404	1:20 (1 + 19)	50	950	Improved Neubauer	Grids 5, 4, 6
16–100	64–400	1:5 (1 + 4)	50	200	Improved Neubauer	Grids 5, 4, 6
2–15	8–60	1:2 (1 + 1)	50	50	Improved Neubauer	Grids 5, 4, 6
<2	<8	1:2 (1 + 1)	50	50	Improved Neubauer or large-volume	All 9 grids Entire slide

Note 1: White-blood-cell pipettes and automatic pipettes that rely on air displacement are not accurate enough for making volumetric dilutions of viscous semen; use positive-displacement pipettes.

Note 2: For diagnostic purposes, semen samples for analysis should be not less than 50 μl in volume, to avoid pipetting errors associated with small volumes.

Note 3: If there are too few spermatozoa per field of view at the recommended dilution, prepare another, lower, dilution. If there are too many overlapping spermatozoa per field of view at the recommended dilution, prepare another, higher, dilution.

Note 4: If a 1 + 19 (1:20) dilution is inadequate, use 1 + 49 (1:50).

Comment 1: If the number of spermatozoa in the initial wet preparation is low (<4 per ×400 HPF: approximately 1×10^6/ml) an accurate sperm number may not be required (see Section 2.10).

Comment 2: For accurate assessment of low sperm concentrations (<2 per ×400 HPF: < approximately 0.5×10^6/ml), it is recommended to use all nine grids of the improved Neubauer chamber (see Section 2.11.1) or a large-volume disposable chamber with fluorescence detection (see Section 2.11.2).

2.8.2 Preparing the dilutions and loading the haemocytometer chambers

- Make the haemocytometer surface slightly damp by breathing on it.
- Secure the coverslip on the counting chambers by pressing it firmly onto the chamber pillars. Iridescence (multiple Newton's rings) between the two glass surfaces confirms the correct positioning of the coverslip. The more lines there are, the better the fit; only one or two lines may indicate problems with variation in chamber depth.

- Use a positive-displacement pipette to dispense the appropriate amount of fixative (see Table 2.3) into two dilution vials.
- Mix the semen sample well (see Box 2.3).
- Aspirate the appropriate volume of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension, (see Table 2.3).
- Wipe the semen off the outside of the pipette tip, taking care not to touch the opening of the tip.
- Dispense the semen into the fixative and rinse the pipette tip by aspirating and expressing the fixative.
- Mix the semen sample well again, and prepare the replicate dilution following the steps above.
- Mix the first dilution thoroughly by vortexing for 10 seconds at maximum speed. Immediately remove approximately 10 μl of fixed suspension, to avoid settling of the spermatozoa.
- Touch the pipette tip carefully against the lower edge of one of the chambers at the V-shaped groove.
- Depress the plunger of the pipette slowly, allowing the chamber to fill by capillary action. The coverslip should not be moved during filling, and the chamber should not be overfilled (when the coverslip may be seen to move) or underfilled (when air occupies some of the chamber area).
- Mix the second dilution, as above, and immediately remove a second 10-μl aliquot. Load the second chamber of the haemocytometer following the steps above.
- Store the haemocytometer horizontally for at least 4 minutes at room temperature in a humid chamber (e.g. on water-saturated filter paper in a covered Petri dish) to prevent drying out. The immobilized cells will sediment onto the grid during this time.

Note 1: Some chambers are constructed with ground-glass pillars; in these, Newton's rings will not appear. Apply about 1.5 μl of water to each ground-glass pillar to hold the coverslip in place (Brazil et al., 2004a), taking care not to introduce water into the counting area.

Note 2: The use of haemocytometer clamps to hold the coverslip in place will ensure a constant depth (Christensen et al., 2005).

Note 3: In very viscous samples, semen can aggregate within the dilution fluid if mixing is delayed by 5–10 seconds. In these cases, vortex the diluted sample for 10 seconds immediately after adding the semen to the fixative.

2.8.3 Assessing sperm numbers in the counting chambers

Sperm number should be assessed in both chambers of the haemocytometer. If the two values agree sufficiently, the aliquots taken can be considered representative of the sample (see Section 2.4.1).

- Examine the haemocytometer with phase-contrast optics at ×200 or ×400 magnification.
- Count at least 200 spermatozoa in each replicate, in order to achieve an acceptably low sampling error (see Box 2.7 and Table 2.2).
- First assess the central grid (number 5 in Fig. 2.7) of one side of the improved Neubauer chamber, row by row.
- Continue counting until at least 200 spermatozoa have been observed and a complete row (of five large squares) has been examined. Counting must be done by complete rows; do not stop in the middle of a row. If 200 spermatozoa are not observed in the five rows of the central grid, continue counting in the rows (of 4 large squares) of the two adjacent grids (nos 4 and 6 in Fig. 2.7).
- Make a note of the number of rows assessed to reach at least 200 spermatozoa. The same number of rows will be counted from the other chamber of the haemocytometer.
- Tally the number of spermatozoa and rows with the aid of a laboratory counter.
- Switch to the second chamber of the haemocytometer and perform the replicate count on the same number of rows (the same volume) as the first replicate, even if this yields fewer than 200 spermatozoa.
- Calculate the sum and difference of the two numbers.
- Determine the acceptability of the difference from Table 2.4 or Fig. A7.1, Appendix 7. (Each shows the maximum difference between the counts that is expected to occur in 95% of samples because of sampling error alone.)
- If the difference is acceptable, calculate the concentration (see Section 2.8.4). If the difference is too high, prepare two new dilutions as described in Section 2.8.2 and repeat replicate counts (see Box 2.10).
- Report the average sperm concentration to two significant figures.
- Calculate the total number of spermatozoa per ejaculate (see Section 2.8.7).

Note 1: If fewer than 200 spermatozoa are found in grids 4, 5 and 6, do not continue to count in grids 1, 2, 3, 7, 8 or 9, since the volume of each row in these grids differs from that of the rows in grids 4, 5 and 6 (see Section 2.7.2). In this case, prepare and assess two lower dilutions. If a 1+1 (1:2) dilution is necessary, proceed as in Section 2.11.

Note 2: Assessing the same chamber twice or assessing both chambers filled from a single dilution is not true replication, as this will not allow detection of errors of sampling, mixing and dilution.

Table 2.4 Acceptable differences between two replicate counts for a given sum

Sum	Acceptable Difference*	Sum	Acceptable Difference*
144–156	24	329–346	36
157–169	25	347–366	37
170–182	26	367–385	38
183–196	27	386–406	39
197–211	28	407–426	40
212–226	29	427–448	41
227–242	30	449–470	42
243–258	31	471–492	43
259–274	32	493–515	44
275–292	33	516–538	45
293–309	34	539–562	46
310–328	35	563–587	47

**Based on the rounded 95% confidence interval.*

Box 2.10 Comparison of replicate counts

The difference between independent counts is expected to be zero, with a standard error equal to the square root of the sum of the two counts. Thus $(N1–N2)/(\sqrt{(N1+N2)})$ should be <1.96 by chance alone for a 95% confidence limit.

If the difference between the counts is less than or equal to that indicated in Tables 2.4 or 2.5 for the given sum, the estimates are accepted and the concentration is calculated from their mean.

Larger differences suggest that miscounting has occurred, or there were errors of pipetting, or the cells were not well mixed, resulting in non-random distribution in the chamber or on the slide.

When the difference between the counts is greater than acceptable, discard the first two values, and prepare and assess two fresh dilutions of semen. (Do not count a third sample and take the mean of the three values, or take the mean of the two closest values.)

This applies to counts of spermatozoa and peroxidase-positive cells (see Section 2.18). For CD45-positive cells (see Section 3.2) and immature germ cells (see Section 2.19), the stained preparations should be reassessed.

With these 95% CI cut-off values, approximately 5% of replicates will be outside the limits by chance alone.

Note: On rare occasions, with inhomogeneous samples, even a third set of replicates may provide unacceptable differences. In this case, calculate the mean of all replicates and note this in the report.

2.8.4 Calculation of the concentration of spermatozoa in semen

It is recommended to calculate and report on the concentration of spermatozoa in semen. Although concentration is not a specific measure of testicular function, it is related to fertilization and pregnancy rates.

The concentration of spermatozoa in semen is their number (N) divided by the volume in which they were found, i.e. the volume of the total number (n) of rows examined for the replicates (20 nl each for grids 4, 5 and 6), multiplied by the dilution factor. That is, $C = (N/n) \times (1/20) \times$ dilution factor.

For 1 + 4 (1:5) dilutions, using grids 4, 5 and 6, the concentration $C = (N/n) \times (1/20) \times 5$ spermatozoa per nl = $(N/n) \times (1/4)$ spermatozoa/nl (or 10^6 per ml of semen).

For 1 + 19 (1:20) dilutions, using grids 4, 5 and 6, the concentration $C = (N/n) \times (1/20) \times 20$ spermatozoa per nl = (N/n) spermatozoa/nl (or 10^6 per ml of semen).

For 1:50 (1 + 49) dilutions, using grids 4, 5 and 6, the concentration $C = (N/n) \times (1/20) \times 50$ spermatozoa per nl = $(N/n) \times 2.5$ spermatozoa/nl (or 10^6 per ml of semen).

2.8.5 Worked examples

Example 1. With a 1 + 19 (1:20) dilution, replicate 1 is found to contain 201 spermatozoa in seven rows, while replicate 2 contains 245 spermatozoa in seven rows. The sum of the values (201 + 245) is 446 in 14 rows and the difference (245–201) is 44. From Table 2.4 this is seen to exceed the difference expected by chance alone (41), so new replicate dilutions are made.

Example 2. With a 1 + 19 (1:20) dilution, replicate 1 is found to contain 220 spermatozoa in four rows, while replicate 2 contains 218 spermatozoa in four rows. The sum of the values (220 + 218) is 438 in eight rows and the difference (220–218) is 2. From Table 2.4 this is seen to be less than that found by chance alone (41), so the values are accepted.

The concentration of the samples for a 1 + 19 (1:20) dilution is $C = (N/n) \times 1.0$ spermatozoa per nl, i.e. $(438/8) \times 1.0 = 54.75$ spermatozoa/nl, or 55×10^6 spermatozoa per ml of semen (to two significant figures).

Note: For 1 + 19 (1:20) dilutions and grids 4, 5 and 6, the concentration is easy to calculate. The total number of spermatozoa counted divided by the total number of rows assessed equals the sperm concentration in 10^6/ml. In the example above the calculation is $(220 + 218)/(4 + 4) = 438/8 = 55 \times 10^6$ spermatozoa per ml of semen.

Example 3. With a 1 + 19 (1:20) dilution, replicate 1 is found to contain 98 spermatozoa in 15 rows (grids 5, 4 and 6), while replicate 2 contains 114 spermatozoa in 15 rows (grids 5, 4 and 6). The sum of the values (98 + 114) is 212 in 30 rows and the difference (114–98) is 16. From Table 2.4 this is seen to be less than that found by chance alone (29), so the values are accepted.

The concentration of the sample for a 1 + 19 (1:20) dilution is $C = (N/n) \times 1.0$ spermatozoa per nl or $(212/30) \times 1.0 = 7.07$ spermatozoa/nl, or 7.1×10^6 spermatozoa per ml of semen (to two significant figures). As fewer than 400 spermatozoa were counted, report the sampling error for 212 spermatozoa given in Table 2.2 (approximately 7%).

Note: In this example, the sample has been overdiluted, since fewer than 200 spermatozoa were found in grids 5, 4 and 6; a 1 + 4 (1:5) dilution would have been more appropriate.

Example 4. With a 1 + 4 (1:5) dilution, replicate 1 is found to contain 224 spermatozoa in four rows, while replicate 2 contains 268 spermatozoa in four rows. The sum of the values (224 + 268) is 492 in eight rows and the difference (268–224) is 44. From Table 2.4 this is seen to exceed the difference expected by chance alone (43), so new replicate dilutions are made.

Example 5. With a 1 + 4 (1:5) dilution, replicate 1 is found to contain 224 spermatozoa in eight rows, while replicate 2 contains 213 spermatozoa in eight rows. The sum of the values (224 + 213) is 437 in 16 rows and the difference (224–213) is 11. From Table 2.4 this is seen to be less than that found by chance alone (41), so the values are accepted.

The concentration of the sample for a 1 + 4 (1:5) dilution is $C = (N/n) \times (1/4)$ spermatozoa per nl or $(437/16)/4 = 6.825$ spermatozoa/nl, or 6.8×10^6 spermatozoa per ml of semen (to two significant figures).

Note: For 1 + 4 (1:5) dilutions the concentration is also simple to calculate but the total number of spermatozoa counted divided by the total number of rows assessed is further divided by 4. In the example above the calculation is $((224 + 213)/(8 + 8))/4 = (437/16)/4 = 27.3/4 = 6.8 \times 10^6$ spermatozoa per ml of semen.

2.8.6 Lower reference limit for sperm concentration

The lower reference limit for sperm concentration is 15×10^6 spermatozoa per ml (5th centile, 95% CI $12–16 \times 10^6$).

2.8.7 Calculation of the total number of spermatozoa in the ejaculate

It is recommended to calculate and report the total number of spermatozoa per ejaculate, as this parameter provides a measure of the capability of the testes to produce spermatozoa and the patency of the male tract. This is obtained by multiplying the sperm concentration by the volume of the whole ejaculate.

2.8.8 Lower reference limit for total sperm number

The lower reference limit for total sperm number is 39×10^6 spermatozoa per ejaculate (5th centile, 95% CI $33–46 \times 10^6$).

2.9 Low sperm numbers: cryptozoospermia and suspected azoospermia

If no spermatozoa are observed in the replicate wet preparations, azoospermia can be suspected. Although it has been suggested that the definition should change (Sharif, 2000; Ezeh & Moore, 2001), azoospermia remains a description of the ejaculate rather than a statement of its origin or a basis for diagnosis and therapy. It is generally accepted that the term azoospermia can only be used if no spermatozoa are found in the sediment of a centrifuged sample (Eliasson, 1981).

However, it should be borne in mind that:

- whether or not spermatozoa are found in the pellet depends on the centrifugation time and speed (Lindsay et al., 1995; Jaffe et al., 1998) and on how much of the pellet is examined;
- centrifugation at 3000*g* for 15 minutes does not pellet all spermatozoa from a sample (Corea et al., 2005); and
- after centrifugation, motility can be lost (Mortimer, 1994a) and concentration will be underestimated (Cooper et al., 2006).

The way these samples are handled depends on whether subjective data on the presence and motility of spermatozoa are sufficient (see Section 2.10) or accurate numbers of spermatozoa are required (see Section 2.11).

2.10 When an accurate assessment of low sperm numbers is not required

If the number of spermatozoa per HPF in the initial wet preparation is low (0 to 4 per ×400 HPF or 0 to 16 per ×200 HPF), several options are available.

2.10.1 Taking no further action

If the number of spermatozoa per ×400 HPF is <4 (i.e. < approximately 1×10^6/ml), it is sufficient for most clinical purposes to report the sperm concentration as $<2 \times 10^6$/ml (to take into account the high sampling error associated with low sperm numbers), with a note as to whether or not motile spermatozoa were seen.

2.10.2 Examination of centrifuged samples to detect spermatozoa

When no spermatozoa are observed in either wet preparation, the sample can be centrifuged to determine if any spermatozoa are present in a larger sample.

- Mix the semen sample well (see Box 2.3). If the sample is viscous, reduce the viscosity as described in Section 2.3.1.1.
- Remove a 1-ml aliquot of semen and centrifuge at 3000*g* for 15 minutes.
- Decant most of the supernatant and resuspend the sperm pellet in the remaining approximately 50 µl of seminal plasma.